EXHIBIT A

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Nonreplicating Viral Vectors as Potential Vaccines: Recombinant Canarypox Virus Expressing Measles Virus Fusion (F) and Hemagglutinin (HA) Glycoproteins

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The development of canarypox virus (CPV) recombinants expressing the hemagglutinin (HA) and fusion (F) glycoproteins of measles virus (MV) is described. Inoculation of the CPV–MV recombinants into avian or nonavian tissue culture substrates led to the expression of authentic MVF and MVHA as determined by radioimmunoprecipitation and surface immunofluorescence. In contrast to avian-derived tissue culture, no productive replication of the CPV recombinant was evident in tissue culture cells derived from nonavian origin. On inoculation of dogs, a species restricted for avipoxvirus replication, the recombinants elicited a protective immune response against a lethal canine distemper virus (CDV) challenge. The level of MV neutralizing antibodies and the level of protection induced against CDV challenge achieved by the host-restricted CPV vector were equivalent to that obtained by vaccinia virus vectors expressing the same MV antigens.

Vaccinia virus is widely used to express antigens from heterologous pathogens providing potential recombinant vaccines for both veterinary and human applications (for recent reviews see (*1, 2*)). In contrast to vaccinia virus, which has a very broad vertebrate host range, other members of the family are restricted for replication to specific hosts. For example, the members of the Avipox genus are naturally restricted for productive replication to avian species (*3, 4*). Two members of the Avipox genus, fowlpox virus (FPV) and canarypox virus (CPV), have recently been engineered to express foreign genes (*5–14*). Successful FPV-based recombinant vaccine candidates have been described for avian influenza (*5*) and Newcastle disease virus (*8, 9, 11, 12*) infections. Although productive replication of avipoxviruses is naturally restricted to avian species, it has been demonstrated that recombinant avipoxviruses inoculated into cells of nonavian origin authentically express foreign genes in the absence of apparent productive viral replication (*6, 13, 14*). Inoculation of an avipox rabies recombinant into a number of species including laboratory rodents, swine, cattle, and horses (*6*) resulted in the induction of rabiesvirus neutralizing antibody. Most importantly, as demonstrated by experiments in mice, cats, and dogs, the level of immunity induced was protective against lethal rabies virus challenge (*6, 14*). In these initial challenge experiments, the 50% protective dose (PD_{50}) of the recombinant FPV was significantly higher than the protective dose obtained with a replication-competent vaccinia virus recombinant expressing the same rabies glycoprotein (*14*). Manipulation of other members of the Avipoxvirus genus, particularly CPV, resulted in recombinant vectors that also induced neutralizing antibodies to rabies virus and provided protective immunity against rabies virus challenge in mice, cats, and dogs (*14*). Significantly, however, the PD_{50} of the CPV/rabies recombinant was approximately 100-fold lower than that of the FPV recombinant and not significantly different from the PD_{50} value obtained with a replication-competent vaccinia rabies recombinant (*14*). In order to determine whether the initial promising result obtained with the CPV/rabies recombinant was peculiar to the rabies glycoprotein immunogen, CPV recombinants expressing extrinsic immunogens from other pathogens were constructed.

Measles virus (MV) and canine distemper virus (CDV) are members of the Morbillivirus genus of the family Paramyxoviridae (*15*). MV causes an acute infectious febrile disease characterized by a generalized macropapular eruption (*16*). Canine distemper is a highly infectious febrile disease of dogs and other carnivores with a mortality rate ranging between 30 and 80%. Dogs surviving CDV infection often have perma-

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nent central nervous system damage (*17*). Both MV and CDV infections are currently controlled by live attenuated vaccines (*17, 18*). The proteins of CDV and MV are structurally similar and share a close serological relationship (*19–21*). In fact, vaccination with MV elicits protection against CDV challenge in dogs (*22, 23*). Expression of MVHA by a vaccinia virus vector also elicits MV neutralizing antibodies and induces a protective immune response in dogs against CDV challenge (*24*).

This report describes CPV recombinants expressing MV hemagglutinin (HA) and fusion (F) glycoproteins. Induction of anti-MV neutralizing antibodies in dogs and protection against live challenge with the related morbillivirus canine distemper virus are described. Further, the efficacy of CP recombinants is compared with that of replication-competent vaccinia virus recombinants.

CPV recombinants expressing MV glycoproteins were developed using a strategy similar to that previously described for FPV (*5, 6*), CPV (*14*), and vaccinia virus (*24*). The strategy for engineering insertion plasmids is illustrated in Figs. 1A and 1B. Insertion of the MVF and MVHA sequences individually led to the development of recombinants CP–MVF (vCP40) and CP-MVHA (vCP50), respectively. To create a single CPV recombinant expressing both MVHA and F, vCP40 was used as a recipient virus for insertion of the HA gene contained in pRW810 (Fig. 1B). vCP57 expresses both MVHA and F genes (Fig. 1B).

To confirm that both proteins were expressed, immunoprecipitation analysis was performed using mono-specific sera directed against either the HA or the F protein. No MVF cross-reactive polypeptides were detectable in uninfected chicken embryo fibroblast (CEF) cells (Fig. 2A, lane a), wild-type virus-infected CEF cells (Fig. 2A, lane b), or CEF cells infected with the HA recombinant (CP–MVHA) (Fig. 2A, lane d). Figure 2A illustrates specific immunoprecipitation of a correctly processed fusion polypeptide from lysates of primary CEF cells infected with the MVF CPV recombinant CP–MVF (lane c) and the MVHA+F double recombinant CP–MVHA+F (lane e). The apparent molecular masses of the radiolabeled polypeptides of 60, 44, and 23 kDa are consistent with the precursor F_0 and the cleavage products, F_1 and F_2. No MVHA-specific products were detected in uninfected cells (Fig. 2B, lane a), wild-type virus-infected cells (Fig. 2B, lane b), or cells infected with the MVF recombinant, CP–MVF (Fig. 2B, lane c). A 75-kDa polypeptide observed in CEF cells infected with the MVHA recombinant CP–MVHA (Fig. 2B, lane d) or the recombinant CP–MVHA+F expressing both MV glycoproteins (Fig. 2B, lane e) is consistent with a fully glycosylated MV hemagglutinin. Immunofluorescence analysis indicated that both MV glycoproteins, HA and F, were present on the plasma membrane of recombinant-infected CEF, VERO, or MRC-5 cells (data not presented).

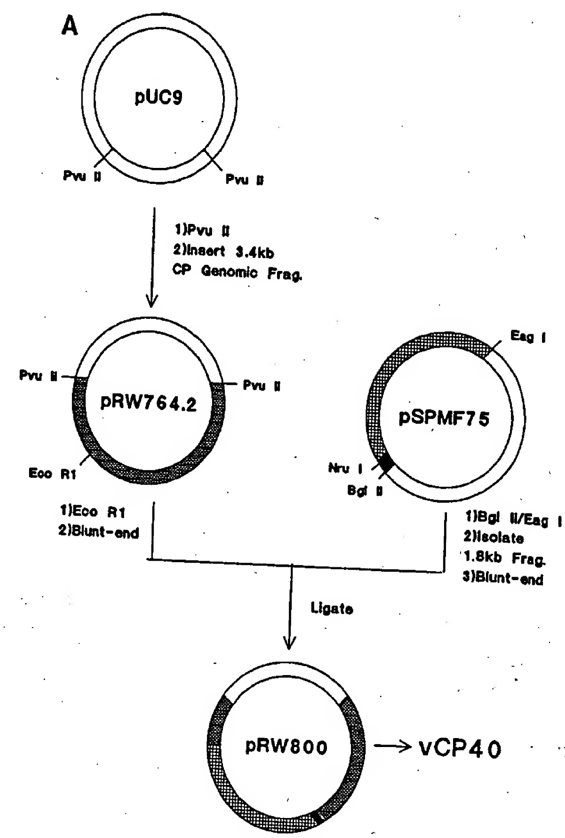

FIG. 1A. Generation of plasmids for insertion of the MVF gene into CPV. The 1.8-kbp blunt-ended *BglII/EagI* fragment from pSPMF75 (*24*) containing the MVF gene juxtaposed 3′ to the vaccinia virus H6 promoter was inserted into the blunt-ended *EcoRI* site of pRW764.2. Plasmid pRW764.2 contains a 3.4-kbp *PvuII* fragment from the CPV genome containing a unique *EcoRI* site. The resultant plasmid containing the MVF gene was designated pRW800 and was used in *in vivo* recombination with CPV as the rescuing virus to generate CP–MVF (vCP40). Plasmid sequences depicted by open areas, CPV sequences by brick-filled areas, MVF by hatched areas, and the vaccinia virus H6 promoter by the solid area.

An important feature of morbillivirus cytopathogenicity is the ability to form syncytia. This fusogenic potential has been functionally assigned, by analogy with other paramyxoviruses, to the amino terminus of the F1 fragment (*25–27*). It has been demonstrated that 293 cells infected at a high multiplicity with an adenovirus recombinant expressing MVF (*28*) or insect cells infected with a recombinant baculovirus expressing MVF produced cell fusion at pH 5.8 (*29*), indicating that in certain systems expression of the fusion protein alone is sufficient for syncytium formation. Expression of both MV glycoproteins HA and F was required for

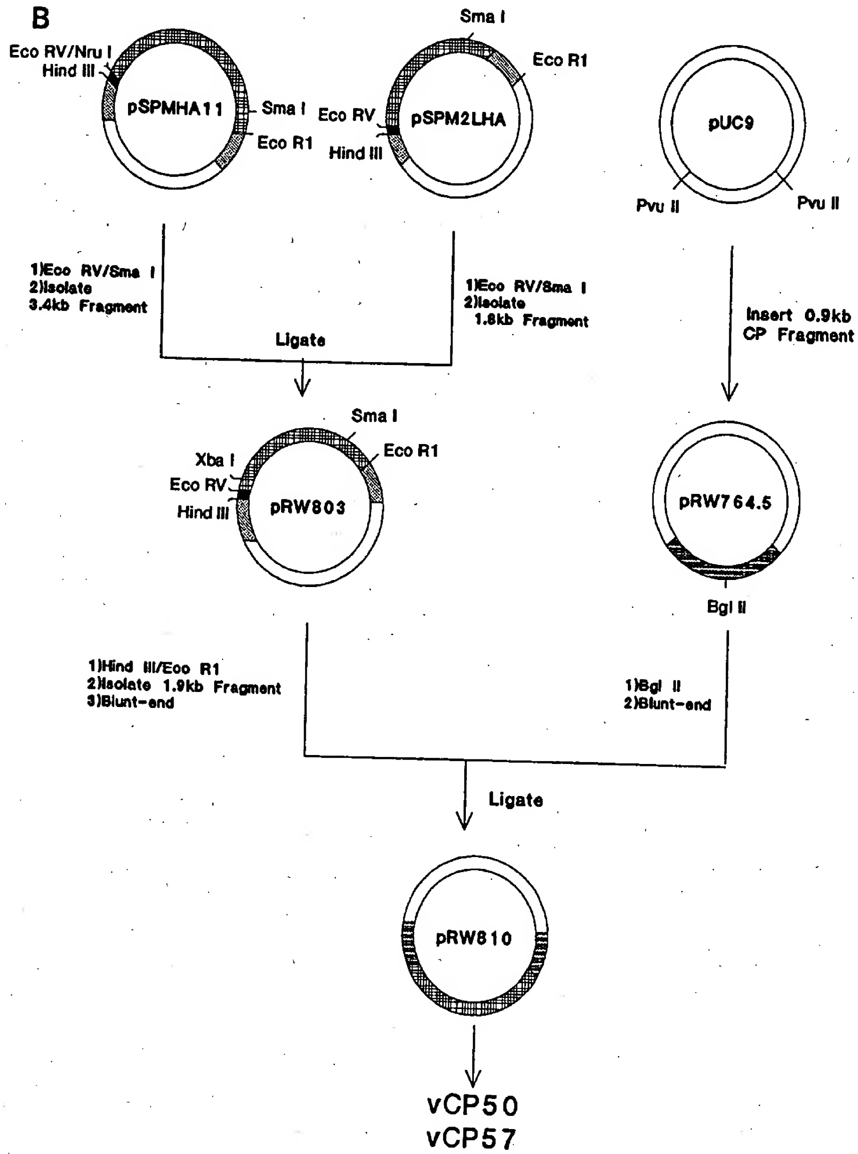

FIG. 1B. Generation of plasmids for insertion of the MVHA gene into CPV and generation of a double CP–MVHA+F recombinant. The 1.8-kbp *EcoRV/SmaI* fragment from pSPM2LHA (*24*) containing the 3'-most 28 bp of the H6 promoter fused in a precise ATG:ATG configuration with MVHA was inserted between the *EcoRV* and *SmaI* sites of pSPMHA11. The resultant plasmid was designated pRW803. A 1.9-kbp *HindIII/EcoRI* fragment of pRW803 containing the H6 promoted MVHA gene was blunt-ended and inserted into the blunt-ended *BglII* site of plasmid pRW764.5. Plasmid pRW764.5 contains a 900-bp *PvuII* fragment of the CPV genome containing a unique *BglII* site. This insertion created plasmid pRW810 which was used in *in vivo* recombination to generate CP–MVHA (vCP50) and CP–MVHA+F (vCP57) using wild-type CPV and CP–MVF (vCP40), respectively, as the rescuing virus. The rescuing virus used in the production of the CP–MV recombinants was the Rentschler strain of CPV, a highly attenuated vaccine strain used for vaccination of canaries. The virus was obtained from Rhone Merieux (Lyon, France). All viruses were grown and titered on primary CEF cells derived from 10- to 11-day-old embryos of SPF origin. Plasmid sequences are depicted by open boxes, CPV sequences by the brick filled boxes, MVHA by hatched boxes, and the vaccinia virus H6 promoter by the solid region.

cell fusing activity when expressed by a vaccinia virus vector (*24, 30*). In order to determine that the MVF glycoprotein expressed by CPV was functionally active, cell fusion assays were performed. Vero cell (monkey kidney cells) monolayers, in which the CPV recombinant does not productively replicate, were infected with 1 PFU per cell of parental or recombinant CPV and examined for cytopathic effects at 18 hr postinfection.

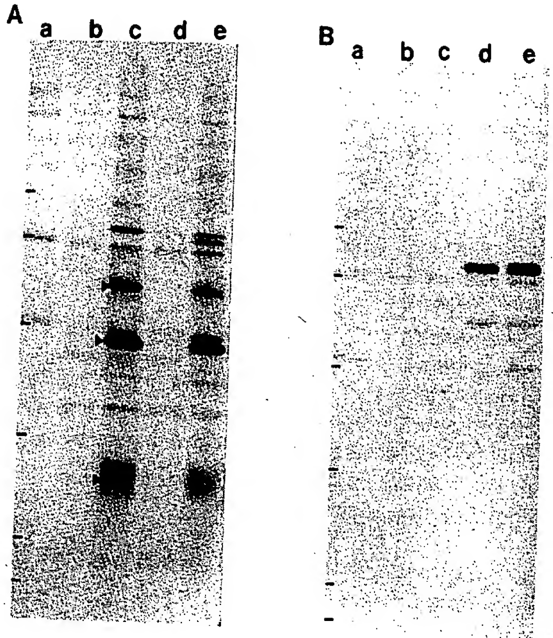

FIG. 2. (A) Immunoprecipitation of F protein from CP–MVF- and CP–MVHA+F-infected CEF cells. Immunoprecipitation was performed as described in (*11*) using a rabbit antiserum directed against a MVF peptide (*24, 29*). Lane a, uninfected CEF cells; lane b, CEF cells infected with parental virus; lane c, CEF cells infected with CP–MVF; lane d, CEF cells infected with CP–MVHA; lane e, CEF cells infected with CP–MVHA+F. The arrowheads to the left of lane c indicate the 60-, 44-, and 23-kDa peptides consistent with F_0, F_1, and F_2. (B) Immunoprecipitation of HA protein from CP–MVHA- and CP–MVHA+F-infected CEF cells. Immunoprecipitation was performed as described in (*11*) using a rabbit antiserum directed against the purified MVHA (*24*). Lane a, uninfected CEF cells; lane b, CEF cells infected with parental virus; lane c, CEF cells infected with CP–MVF; lane d, CEF cells infected with CP–MVHA; lane e, CEF cells infected with CP–MVHA+F. Marks to the left indicate migration distances for standard proteins with molecular weights (from the top) of 97.4, 68, 43, 29, 18.4, and 14.3 kDa.

No cell fusing activity was evident in Vero cells inoculated with parental (Fig. 3B), CP–MVF (Fig. 3C), or CP–MVHA (Fig. 3D) viruses. However, when Vero cells were inoculated with CP–MVHA+F expressing both MVF and MVHA measles glycoproteins (Fig. 3F) or were coinfected with the individual recombinants CP–MVF and CP–MVHA (Fig. 3E), efficient cell fusing activity was evident, consistent with published reports (*24, 30*).

Previous studies had indicated that a vaccinia virus vector expressing either the MVHA or the MVF protein could protect dogs against a lethal challenge with CDV (*24*). It was of interest to know whether host-restricted CPV expressing the same MV glycoproteins would induce a protective immune response. Ten-week-old specific pathogen-free beagle dogs were inoculated with parental CPV or recombinant CP–MVF, CP–MVHA, or CP–MVHA+F viruses as described in Table 1. For comparison, one dog was inoculated in the same regimen with each of the single vaccinia recombinant viruses, VV–MVF or VV–MVHA, or a mixture of both VV–MVHA and VV–MVF recombinants (*24*). The results are shown in Tables 1, 2, and 3. No adverse reactions to vaccination were noticed in any of the dogs during the course of the experiment. The two dogs immunized with parental CPV and the two nonimmunized control dogs showed severe disease after challenge. All four dogs became depressed, showed elevated body temperature, weight loss, lymphopenia, and severe dehydration. The disease state progressed and euthanasia was warranted (Tables 2, 3). Dogs immunized with CDV–Rockborn developed neutralizing antibodies against CDV (results not shown) but not against MV prior to challenge (Table 1) and survived lethal CDV challenge, symptom free (Tables 2, 3). Dogs immunized with MV developed neutralizing antibodies to MV (Table 1) but not to CDV prior to challenge and survived challenge with mild signs of infection (Tables 2, 3). Neither of the two dogs inoculated with the CPV recombinant, CP–MVF, nor the dog (9/9) receiving the vaccinia F recombinant, VV–MVF, developed MV neutralizing antibody after two inoculations (Table 1). It has previously been shown that fusion antibodies have a low neutralizing activity in *in vitro* tests (*31*), although Malvoisin and Wild (1990) (*32*) have recently shown that anti-F monoclonal antibodies with neutralizing activity can be isolated. Despite the absence of detectable MV (Table 1) or CDV (not shown) neutralizing antibody, the dogs survived challenge with CDV, although with signs of disease (Tables 2, 3). Dogs inoculated with CP–MVHA, CP–MVHA+F, VV–MVHA, or coinoculated with VV–MVF and VV–MVHA developed significant MV neutralizing antibody after a single inoculation, and the titer was boosted on reinoculation with the CPV recombinants (Table 1). No CDV neutralizing antibodies were detected in any of these dogs (data not shown). Nevertheless, these dogs survived virulent CDV challenge with minor signs of CDV infection (Tables 2, 3).

These results demonstrate the protection of dogs from live CDV challenge by vaccination with host-restricted CPV recombinants expressing the MVHA and MVF glycoproteins and corroborate our previous findings with vaccinia virus vectors (*24*). In addition, the results indicate that the host-restricted nonreplicating CPV vectors expressing the MV glycoproteins elicit MV neutralizing antibody titers equivalent to those induced by vaccinia virus vectors expressing the same MV glycoproteins. This provides another observation demonstrating that immune responses and protection against

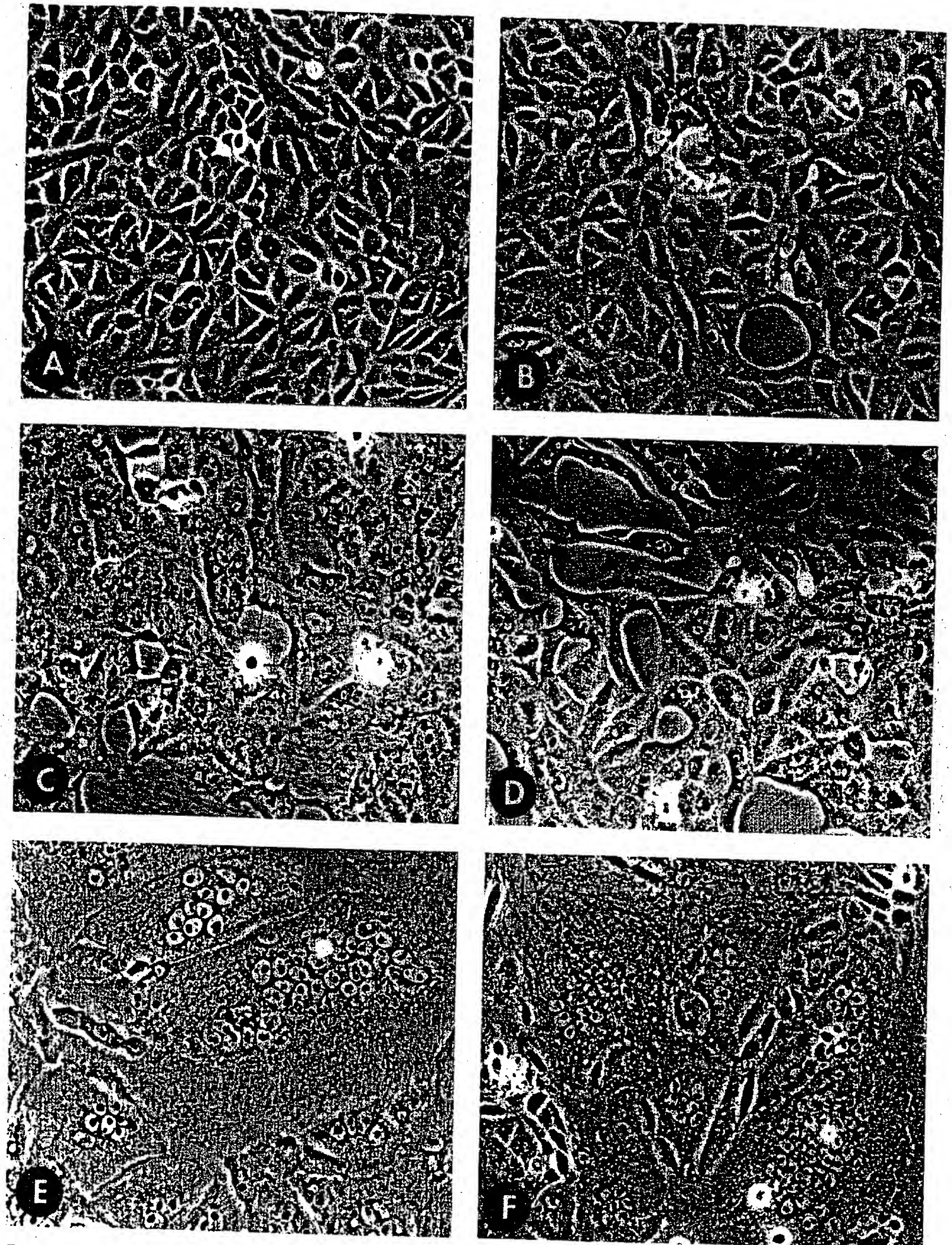

FIG. 3. Fusogenic activity of CPV recombinants expressing MV glycoproteins. Cell fusion experiments were performed as described in (24). Vero cell monolayers (uninfected, A) were inoculated with 1 PFU per cell of CP parental virus (B), CP–MVF (C), CP–MVHA (D), CP–MVF and CP–MVHA (E), or CP–MVHA+F (F). The cytopathic effect induced was examined microscopically at 18 hr postinfection using a Nikon Diaphot inverted microscope equipped with phase-contrast optics using a 20× objective lens and photographed with Kodak Technical Pan film ASA 25.

TABLE 1

MEASLES VIRUS NEUTRALIZING ANTIBODY INDUCED IN DOGS INOCULATED WITH POXVIRUS RECOMBINANTS EXPRESSING MEASLES FUSION AND/OR HEMAGGLUTININ GLYCOPROTEINS

Immunogen	Dog no.	Days postvaccination					
		0[a]	7	14	21[b]	28	35[c]
CPV	9/1	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
	9/2	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
CP–MVHA	9/3	<1.0	2.7[d]	2.9	3.2	4.4	4.1
	9/4	<1.0	1.7	2.7	2.7	3.9	3.9
CP–MVF	9/5	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
	9/6	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
CP–MVHA + F	9/7	<1.0	2.0	2.7	2.5	3.9	3.6
	9/8	<1.0	1.0	2.2	2.0	3.6	3.4
VV–MVF	9/9	<1.0	<1.0	<1.0	1.0	1.0	1.0
VV–MVHA	9/10	<1.0	2.9	2.5	3.2	3.4	3.4
VVMVF + VV–MVHA	9/11	<1.0	1.3	2.9	2.9	2.9	2.9
MV	9/12				<1.0	2.5	2.5
Control	9/13						<1.0
	9/14						<1.0
CDV–Ro	9/15				<1.0	<1.0	<1.0

Note. Fifteen 10-week-old specific pathogen-free beagle dogs from the James A. Baker Institute colony were studied. Blood samples were collected at the initiation of the experiment and repeatedly thereafter. Four groups of two dogs were immunized via a subcutaneous inoculation (1×10^8 PFU in 1 ml). The first group received parental CPV only. The second group received CPV with an insert for the MVHA protein (CP–MVHA). The third group received CPV with an insert for the MVF protein (CP–MVF), and the fourth group received a CPV recombinant expressing both HA and F genes (CP–MVHA + F). In addition, one dog each was inoculated subcutaneously at the same time intervals with 1×10^8 PFU in 1 ml of vaccinia virus with an insert for the HA protein of MV (VV–MVHA), or the F protein of MV (VV–MVF) or a combination of both VV–MVHA and VV–MVF viruses (*24*). One control dog received 10^5 tissue culture infectious doses ($TCID_{50}$) of the attenuated Edmonston strain of MV intramuscularly (1 ml) and one control dog received 10^4 $TCID_{50}$ of the attenuated Rockborn strain of CDV subcutaneously 2 weeks before challenge with virulent CDV. Two dogs remained uninoculated. Blood samples for serological tests were collected before vaccination and at weekly intervals until the time of challenge and on days postchallenge (dpc) 7, 12, and 21. Serum neutralization titers were assessed as described in (*34*).

[a] Day of first immunization.

[b] Day of second immunization with same inoculum and route. First immunization with MV and CDV–Ro.

[c] Day of challenge by intranasal inoculation of 1 ml of 10^4 $TCID_{50}$ of the virulent Snyder Hill strain of CDV.

[d] Serum neutralization titers assessed as described in (*34*) and expressed as $\log_{10}$ of last antibody dilution showing complete neutralization of infectivity in a microtiter assay.

a lethal virus challenge can be obtained using a host range-restricted CPV vector which are equivalent to the immune responses obtained with a replication-competent vaccinia virus vector expressing the same extrinsic immunogens (*14, 24*).

The observations reported here and elsewhere raise a number of intriguing issues (*6, 14*). It is surprising to find that a host range-restricted avipoxvirus vector can induce titers of neutralizing antibody and protection against lethal virus challenge equivalent to antibody titers and protection obtained with a vaccinia virus vector with a broad host range. The basis of this is currently unknown but contrasts with previous thinking that high level expression of the extrinsic immunogen and replication of the vector with concomitant amplification of the foreign antigenic mass were considered to be important features. It is clear that productive replication of the vector per se is apparently not essential for the induction of protective immunity. Further, not all nonreplicating vectors work to the same degree since CPV-based vectors were shown to be approximately 100 times more efficient than FPV-based vectors in inducing immunity to rabies (*14*). It is interesting to speculate that immunomodulating factors exist within the avipoxvirus vectors themselves. However, the rapid early expression of the extrinsic immunogen, presentation of the extrinsic immunogen at high density on the plasma membrane of the infected cell, and the failure of the avipox vector to shut off host macromolecular synthesis or to complete its replication cycle may be important. Under these conditions the immune response may be favorably skewed toward the extrinsic immunogen and away from competing antigens of the vector.

TABLE 2

Clinical Course of Infection of Dogs Immunized with Poxvirus/Measles Recombinants Following Virulent CDV Challenge

Dog no.	Immunogen	Days post-CDV-SH challenge: 4	6	8	10	12
9/1	CP	L, A	L, A	SD, A	SD, A, C	
		T		T, C	D, E	
9/2	CP	L, A, T	L, A	SD, A, T	SD, A, C	
					D, E	
9/3	CP–MVHA		T			
9/4	CP–MVHA		T			
9/5	CP–MVF	L, A, T	L, A, C	SD, A, C	SD, A, C	I
			DI	DI		
9/6	CP–MVF	L, A, T	L, A	I		
			DI, T			
9/7	CP–MVHA + F		T			
9/8	CP–MVHA + F	T	T		T	
9/9	VV–MVF	T	L, H	SD, A, C	I	
9/10	VV–MVHA					
9/11	VV–MVF + VV–MVHA					
9/12	MV					
9/13	Control	L, A, T	L, A, T	SD, A, C	SD, A, C	
					D, E	
9/14	Control	L, A, T	L, A	SD, A	SD, D	
				DI	DI, E	
9/15	CDV–Ro					

Note. The immunization schedule is described in the legend to Table 1. Dogs were challenged by intranasal inoculation of 1 ml of tissue culture fluid containing 10^4 $TCID_{50}$ of the Snyder Hill strain of virulent CDV. The clinical reactions of the dogs were monitored by daily observations with recording of body temperature. L, Lethargic; A, anorectic; SD, severely depressed; C, conjunctivitis; D, dehydrated; E, euthanized; DI, diarrhea; I, improved; T, elevated body temperature above 39.5°. Empty spaces designate no clinical symptoms (no abnormality was noted).

TABLE 3

Laboratory Confirmation of Infection of Immunized Dogs Following Challenge with Canine Distemper Virus

Virus	Dog	Days post-CDV-SH challenge: 3	5	7	10
CP	9/1	0.1[a], V[b]	Ly[c], V	0.9, Ly, V	1.4, Ly, V
CP	9/2	Ly, V	Ly, V	0.9, Ly, V	1.9, Ly, V
CP–MVHA	9/3		Ly	0.9, Ly, V	0.2
CP–MVHA	9/4			0.6, Ly	
CP–MVF	9/5	0.3	Ly, V	1.2, Ly, V	1.9, Ly
CP–MVF	9/6	0.1	Ly, V	1.3, V	1.3
CP–MVHA + F	9/7		Ly	0.7	0.5
CP–MVHA + F	9/8		V	0.4, Ly, V	0.5, Ly
VV–MVF	9/9	V	Ly, V	1.5, Ly, V	1.7, Ly, V
VV–MVHA	9/10	0.1		0.1	0.3
VV–MVF + VVMVHA	9/11	0.1	Ly	0.7	0.5
MV	9/12		Ly, V	0.3	0.4
Naive	9/13	0.2	Ly, V	1.2, Ly, V	NT, Ly, V
Naive	9/14	0.3, Ly	Ly, V	0.9, Ly, V	2.1, Ly
CDV–Ro	9/15				

Note. Immunization and challenge schedules are described in the legends to Tables 1 and 2. Weight gains or losses were recorded biweekly. Circulating blood lymphocytes were counted before challenge and on dpc 3, 5, 7, and 10. Virus isolation from buffy coat cells by cocultivation with dog lung macrophages was attempted on dpc 3, 5, 7, and 10 as described in (*33*).

[a] Weight loss in kilograms.

[b] V, Challenge virus was isolated.

[c] Ly, Lymphopenia evidenced by a total lymphocyte count below 2×10^3 lymphocytes per mm^3.

Empty spaces designate normal measurements.

The absence of apparent replication of the avipox vector would suggest a significant degree of safety since the vector should not be able to disseminate within the vaccinated individual or to nonvaccinated contacts or the general environment. It will be interesting to see whether such recombinants would be useful in the vaccination of young pups for the prevention of CDV infections and, more specifically, whether such recombinants would be useful for vaccinating infants at an early age against MV infection, thus addressing one of the major deficiencies of the currently available attenuated measles virus vaccine.

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